Journal of Pharmaceutical Research International

32(16): 75-86, 2020; Article no.JPRI.59708
ISSN: 2456-9119
(Past name: British Journal of Pharmaceutical Research, Past ISSN: 2231-2919, NLM ID: 101631759)

Targeting the Secreted Aspartic Proteinase (SAP-1) Associated with Virulence in *C. albicans* by *C. cassia* Bio-compounds: A Computational Approach

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Authors' contributions

This work was carried out in collaboration among all authors. Author ASSG designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors AR and VP managed the analyses of the study. Author AR managed the literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JPRI/2020/v32i1630651
Editor(s):
(1) Dr. Sung-Kun Kim, Northeastern State University, USA.
Reviewers:
(1) Marwa Derma, Dermoscope, Al-Azhar University, Egypt.
(2) Mohamed E. E. Shams, Mansoura University, Egypt.
Complete Peer review History: http://www.sdiarticle4.com/review-history/59708

Original Research Article

Received 28 May 2020
Accepted 03 August 2020
Published 24 August 2020

ABSTRACT

Evaluation of the drug ligand interactions between the *C. cassia* bio-compounds with the *SAP-1* in *C. albicans* to explore the inhibitory medicinal potential of *C. cassia* bio-compounds by a computational approach is performed in the present investigation.
Antimicrobial assay was done using agar well diffusion method with the crude aqueous and ethanolic extracts of the dried barks of *C. cassia* against *C. albicans*. 2D & 3D structures of the active bio-compounds of *C. cassia* were optimized and the 3D structure of *SAP-1* was retrieved from the PDB data bank. *In-silico* inhibitory potential of the selected *C. cassia* biocompounds against *SAP-1* was done by Auto Dock 2.0 and was visualized with Accelrys discovery studio visualizing tool with the assessment of the molecular properties of the ligands against *SAP-1* by molinspiration calculations and further assessment for their drug likeliness. *In-vitro* analysis showed

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a promising anti-fungal activity of *C. cassia* extracts against *C. albicans*. Cinnamoyl E-acetate and Eugenyl acetate seem to possess promising inhibitory effect to target *SAP-1* with a least binding energy of –5.33 and -5.21 Kcal/mol with four hydrogen bonds respectively. Molinspiration assessments showed zero violations for all the *C. cassia* compounds with the TPSA scores of <140 Å towards the best oral bioavailability. The findings of the study emphasize that cinnamaldehyde, cinnamoyal acetate and eugenol from *C. cassia* seem to possess a promising inhibitory effect against *SAP-1* of *C. albicans* suggesting the medicinal value of the spice against *SAP-1.*

Keywords: C. albicans; SAP-1; C. cassia; cinnamaldehyde; cinnamoyal acetate.

1. INTRODUCTION

The field of alternative medicine is well established in a developing country like India, and with the application of many herbs and spices in the treatment of various systemic ailments, *Cinnamomum cassia* and its bio-compounds has spurred renewed interest for its medicinal value in recent years. The potent anti-fungal property of *C. cassia* compounds when applied alone or in combination with other anti-fungal drug of choice like amphotericin B is noticeable [1,2]. The phytochemical analysis of the *C. cassia* has shown the presence of various vital bio-active compounds amidst which cinnamaldehyde, cinnamoyl acetate and eugenol seem to possess potent anti-microbial properties. However, the medicinal role of *C. cassia* compounds is yet to explore against candidal yeast infections. *Candida albicans* is considered as a commensal yeast which can colonize on the mucosal layers of the oral cavity, gastro-intestinal tract, urinary tract etc., in healthy individuals. In traumatized conditions, *C. albicans* may induce systemic infections through hematogenous spread [3]. An array of factors is associated with candidal virulence, colonization, modifications of host immune response etc., during the establishment of the candidal infection [4]. The frequency of these virulence factors may vary based on the site, stage and nature of the host immunity [5]. Amidst many virulence proteins secreted aspartic peptidases [SAP's] with a group of ten acidic hydrolases contribute their key role in candidal virulence. SAP's are mainly involved in fungal nutritional metabolism facilitating the candidal growth [6]. SAP's do play a potent role in the inactivation of the complement components and certain peptides released by the host upon fungal invasion such as histatin/cathelicidin and kininogens[7,8] and possess anti-phagocytic property promoting[9] the fungal adhesion to the host tissues [10]

Most SAP's in *candida* are synthesized as zymogens, or as inactive precursors, which provide protection from proteolysis. The zymogen is converted into an active enzyme by a change in pH, which is sufficient to enhance the autocatalytic conversion mechanism [11]. SAP's are generally sensitive to pepstatin with some reports suggesting its insensitiveness to the same but sensitive to diazoacetic-d-norleucine methyl ester (DAN) and 1,2-epoxy-3-(p-nitrophenoxy) propane (EPNP) in the presence of copper ions . With such variations against chemicals and with the emergence of candidal resistance to anti-fungal drugs, targeting SAP's with natural bio-active compounds would be a novel method to curb the progression of candidal infection in the mucosal tissues.

The main mechanism behind the medicinal antifungal property of the *C. cassia* bio-compounds was associated with the severe damage in the fungal cell membrane and cell walls, leading to morphological deformations and collapse and deterioration of fungal structures[12]. In addition, the inhibitory activity against *SAP* of *Candida* has been documented with the bio-compounds from *Cinnamomum verum* [13]. However, no studies have reported the inhibitory effect against *SAP-1* from *C. cassia*. This present investigation is thus aimed to explore the medicinal value of *C.cassia* biocompounds viz., cinnamaldehyde, cinnamoyl acetate and eugenol in targeting the *SAP-1* of *C. albicans* in comparison with itraconazole through a computational approach to progress further with experimental research.

2. METHODS

2.1 Samples

Dried barks of *Cinnamomum cassia* were purchased commercially and ground into fine powder. Crude extracts were prepared using distilled water and ethanol by mixing 500 mg of the dried powder in 50 ml of the solvents (w/v) as three individual replicates. The mixture was allowed to stand in an orbital shaker for 24 hrs at

room temperature. Crude extracts were then filtered through Whatman filter paper which was further subjected for rotary evaporation and the final extract were stored in dark glass vials and was covered with aluminium foil until use.

2.2 Antifungal Bioassay

Fresh cultures of *Candida albicans* (ATCC 10231) was inoculated onto Saboraud's dextrose broth (HiMedia, Mumbai) and was incubated at 37°C for 2hrs. Broth suspensions were adjusted to 0.5 McFarland's standards and lawn cultures were made onto sterile Saboraud's dextrose agar plate. Wells were cut using sterile agar cutter and 50 µl of the extracts at a concentration of 100 mg/ml and 50 mg/ml each of the aqueous and ethanol solvents were added in to the wells. Itraconazole was used as the control. The plates were incubated at 37°C for 24 hrs and the anti-fungal activity was interpreted by measuring the zone of inhibition around the wells using the HiMedia Antibiotic zone measurement scale. The bio-assay was done in triplicates and the results were recorded.

2.3 Retrieval of SAP and Protein Optimisation

The crystal structure of secreted aspartic proteinase 1 (SAP-1) was retrieved from Research Collaboratory for Structural Bioinformatics (RCSB) protein data bank (http://www.rcsb.org/pdb). Hydrogen atoms, solvation parameters and fragmental volumes to the protein were added and electronic charges were assigned to the protein atoms using Kollman united atoms force field by using Auto Dock Tool (ADT) –1.5.6.

2.4 Ligand Preparation and Optimization

Using Chemsketch Software the structures of the cinnamaldehyde, cinnamoyl acetate, eugenol and the control itraconazole were drawn together with the generation of their 3D structures and optimization. The selected ligands were retrieved in SDB format which were further saved in.mol file followed by the subsequent conversion using open babel molecular converter program [14] and were saved in PDB format.

2.5 Docking Simulations and Interpretations

The docking analysis to interpret the affinity between cinnamaldehyde, cinnamoyl acetate, eugenol and the control itraconazole against Sap-1 of *C. albicans* was achieved by auto-dock tool with the intermediary steps such as pdb.qt files for the proteins and the ligands. Using graphical user interface program Auto-Dock tool (ADT) the grid box creation was completed. Prior preparation of the grid map using the grid box with a grid size of 126x126x126 xyz points was done. Further using Lamarckian genetic algorithm (LGA), docking simulation was achieved by setting the initial position, orientation and torsions of the ligand molecules in a random position. 10 different runs set to terminate after a maximum of 250000 energy evaluations was used for each docking experiment with the population size set at 150. A translational step of 0.2 Å, quaternion and torsion steps of 5 were applied for each dock. The most favorable free energy of binding is achieved by clustering the results > 1.0 Å in positional root-mean-square deviation (RMSD) [15]. Finally, the pose was extracted and aligned with the receptor structure with the lowest binding energy or binding affinity for final analysis.

2.6 Molinspiration Assessment of the Molecular Properties of the Selected Compounds

The physiochemical and the pharmacological properties of *C. cassia* biocompounds such as logP, hydrogen bond donor and acceptor characteristics, molecular size and rotatable bonds were predicted by molinspiration server [16]. Based on the Lipinsky's rule of five [17] characterization of the absorption, distribution, metabolism and elimination (ADME) of the selected compounds and estimations of the molecular properties of the selected ligands was assessed. Membrane permeability and bio-availability was also evaluated towards the medicinal value of the spice *C. cassia*.

2.7 Docking Visualization

The protein-ligand interactions like hydrogen bonding and other non-bonded energies between the cinnamaldehyde, cinnamoyl acetate, eugenol and the control itraconazole against *Sap-1* of *C. albicans* were visualized using Accelrys Discovery Studio Visualizer software. The relative stabilities were evaluated using their molecular dynamics, binding affinities, energy simulations with further docking score assessments.

3. RESULTS AND DISCUSSION

3.1 Anti-fungal Bioassay

Table 1 shows the promising antifungal activity of the crude aqueous and ethanol extracts of *C. cassia* with a mean of 15.6 and 13.3 mm for the aqueous extracts at 100 mg/ml and 50 mg/ml concentration. Similarly, the ethanol extracts recorded a zone size of 17 mm and 15.6 mm at 100 mg/ml and 50 mg/ml.

3.2 Structure Retrieval of the SAP-1 Protein from *C. albicans*

The crystal structure of serine aspartate protease *(SAP-1)* from *Candida albicans* (strain SC5314) is downloaded from PDB database and its structure id was documented as 2QZW-A-chain (Fig. 1). Removal of the water molecules and final stage merging of hydrogen atoms to the receptor molecule was successful. The 3D structure of *SAP-1* was visualized using RASMOL with the analysis of pink colour indicating the alpha-helix, yellow arrow indicating the beta sheets and white colour indicating the turns (Fig. 2).

3.3 Structure Retrieval of the *C. cassia* Compounds (the Ligands)

The ligand optimization was achieved using ACD Chemsketch and retrieved in a compatible format using Open Babel molecular converter tool. The retrieved 2D and 3D structures of the ligands and its SMILES format are shown in Table 2.

Fig. 1. *SAP-1* protein retrieval from PDB sowing the ID of the selected chain for the study

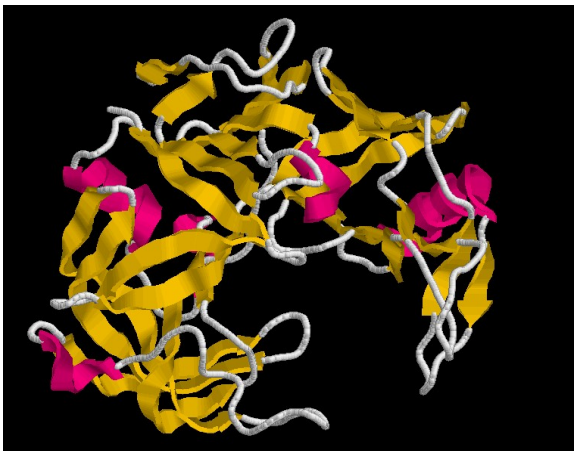
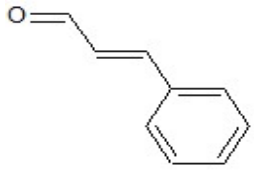
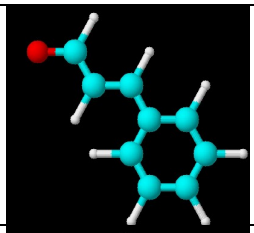
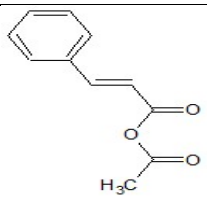
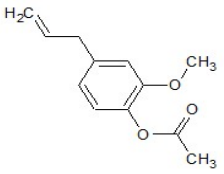
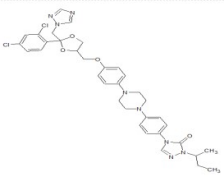


Fig. 2. Showing the 3D structure of Serine aspartate proteinase -1 using RASMOL

Table 1. Antifungal activity of the crude aqueous and ethanol extracts of *C. cassia* against *C. albicans*

Studied strains	Extracts under study	Zone interpretation (100 mg/ml) in mm			Mean	Zone interpretation (50 mg/ml) in mm			Mean
C.albicans		*Test 1*	*Test 2*	*Test 3*		*Test 1*	*Test 2*	*Test 3*	
(ATCC 10231)	Aqueous	16	15	16	15.6	14	14	12	13.3
	Ethanol	18	17	16	17	15	16	16	15.6

Table 2. 2D and 3D structures and SMILES format of the selected bio-compounds from *C. cassia* under study

Compounds	2D	3D	SMILES
(E)-cinnamaldehyde			C1=CC=C(C=C1)C=CC=O
Cinnamoyl (E)-acetate			CC(=O)OC(=O)C=CC1=CC=CC=C1
Eugenyl acetate			CC(=O)OC1=C(C=C(C=C1)CC=C)OC
Itraconazole			CCC(C)N1C(=O)N(C=N1)C2=CC=C(C=C2)N3CCN(CC3)C4=CC=C(C=C4)OCC5COC(O5)(CN6C=NC=N6)C7=C(C=C(C=C7)Cl)Cl

3.4 Molinspiration Estimation of *C. cassia* Biocompounds

The bioactivity scores predicted for cinnamaldehyde, cinnamoyl acetate, eugenol and the control itraconazole against *Sap-1* is scored and tabulated in Table 3. Molecular properties were calculated on the based on the Lipinski's rule of five and its components. From the molinspiration results, the n-violation values of bioactive compounds (E)-cinnamaldehyde, cinnamoyl (E)-acetate, and eugenyl acetate are zero satisfying Lipinski's Rule of 5, whereas the control drug itraconazole shows 3 violations. TPSA was < 140 Å for all the compounds thus indicating its higher absorption and promising oral bio-availability.

3.5 Docking Analysis of the *C. cassia* Compounds against SAP-1 of *C. albicans*

The best conformers were selected using LGA based on the best ligand-receptor structure from the docked structure based on the lowest energy and minimal solvent accessibility. Accelrys discovery studio visualizing tool of the hydrogen bond interactions in stick model between the

Table 3. *Molinspiration calculations of C. cassia* biocompounds

Compounds	M.wt	Mol formula	Hydrogen Bond Donor	Hydrogen Bond Acceptor	miLogP	Rotatable bonds	nViolations	TPSA (Å)	Volume	N atoms
(E)-Cinnamaldehyde	132.162	C_9H_8O	0	1	2.48	2	0	17.07	130.44	10
Cinnamoyl (E)-acetate	190.198	$C_{11}H_{10}O_3$	0	3	2.61	4	0	43.38	174.97	14
Eugenyl acetate	206.241	$C_{12}H_{14}O_3$	0	3	1.90	5	0	35.54	198.65	15
Itraconazole	705.641	$C_{35}H_{38}Cl_2N_8O_4$	0	12	5.32	11	3	104.73	607.90	49

cinnamaldehyde, cinnamoyl acetate, eugenol and the control itraconazole against *Sap-1* of *C.albicans* is given in Fig. 3. The amino acids of *SAP-1* binding with the bioactive compounds, namely, (E)-cinnamaldehyde, cinnamoyl (E)-acetate, Eugenyl acetate and control ligand itraconazole showed the binding energy of –4.91 Kcal/mol with one hydrogen bond interaction, –5.33 Kcal/mol with 4 hydrogen bond interaction, –5.21 Kcal/mol with one hydrogen bond interaction and –8.05 Kcal/mol with one hydrogen bond interaction, respectively. The torsional energy and the docking scores between the drug and ligands are given in Table 4a, 4b, 4c, 4d and 4e. The docking results shows *C. cassia* bio-compounds with better binding energy and bonding with the target receptor with *SAP-1* in comparison with itraconazole. It was also evident that compounds cinnamoyl (E)-acetate and Eugenyl acetate are more potent in targeting *SAP-1*.

Table 4a. Binding energy between *SAP-1* and cinnamaldehyde

SAP-1		(E)-cinnamaldehyde	Distance (Å)	Docking Energy (Kcal/Mol)
Residue	**Atom**			
TYR252	NH	O	1.86	–4.91

Table 4b. Binding energy between *SAP-1* and cinnamoyl acetate

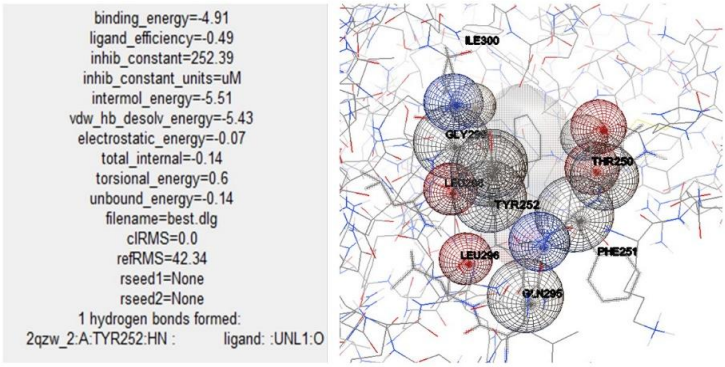
SAP-1		Cinnamoyl (E)-acetate	Distance (Å)	Docking Energy (Kcal/Mol)
Residue	**Atom**			
THR222	N	O	2.81	–5.33
THR222	N	O	3.16	
THR222	OG1	O	3.03	
TYR225	OH	O	3.05	

Table 4c. Binding energy between *SAP-1* and eugenyl acetate

SAP-1		Eugenyl acetate	Distance (Å)	Docking Energy (Kcal/Mol)
Residue	**Atom**			
THR222	N	O	2.63	–5.21
THR222	N	O	3.13	
THR222	OG1	O	2.93	
THR222	OG1	O	3.02	

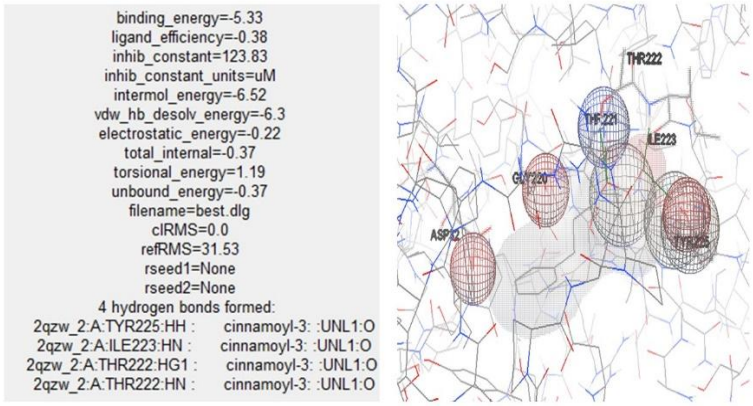
Table 4d. Binding energy between *SAP-1* and itraconazole

SAP-1		Itraconazole	Distance (Å)	Docking Energy (Kcal/Mol)
Residue	**Atom**			
GLY85	N	O	3.00	–8.05

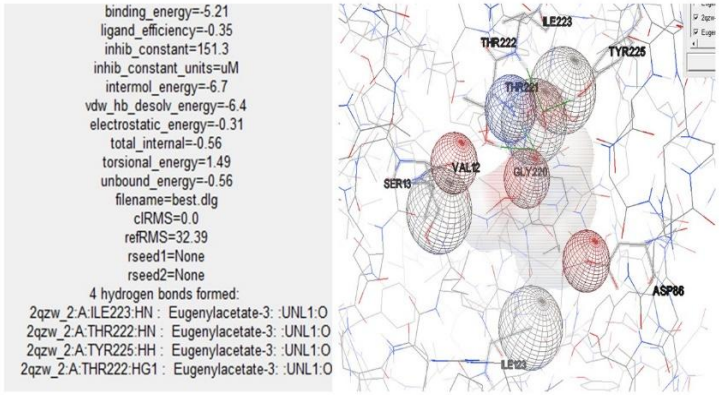


a.

b.



c.



d.

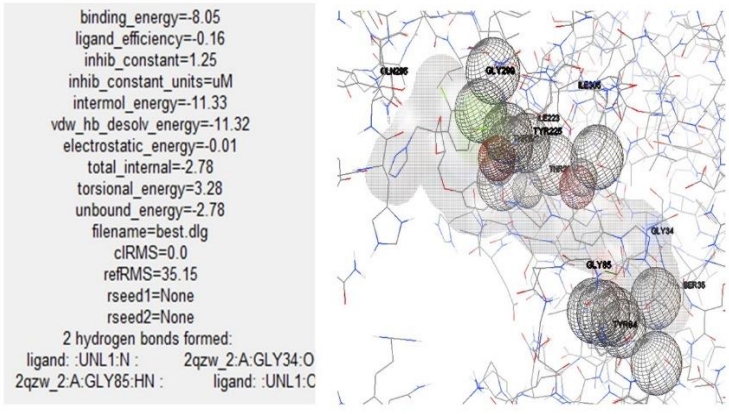


Fig. 3. Acceryls discovery studio visualization of the hydrogen interactions between *SAP-1* with a. E-cinnamaldehyde, b. cinnamoyl E-acetate c. eugenyl acetate d.itraconazole

C. cassia and its bio-compounds, being phytochemically rich in tannins, flavonoids and alkaloids, are best known for its anti-fungal potential [18]. Targeting secreted aspartic proteinase *(SAP)* activity in *C. albicans* exhibited by a multi-gene SAP family, and playing a vital role in varying the extent of virulence in candidal infections[19] , the present investigation would be novel in exploring the medicinal value of *C. cassia* compounds against candidal infections. In the emergence of resistant forms of *Candida* sp., *SAP* can be considered as the best target for

novel antifungal agents especially for *C. cassia* bio-compounds. In the present study, we performed a preliminary in-vitro antifungal bio-assay using well diffusion assay which has documented a promising inhibitory effect of the crude extracts of *C. cassia* against *Candida albicans*. The study has its own limitation of lacking the in-vitro bio-assay for the inhibitory activity of the bio-compounds against SAP protein due to cost effective procedures. Thus we applied the molecular docking protocols for further inhibitory evaluations. The best fit of the bio-compounds of *C. cassia* for its medicinal value with the *SAP-1* of *C. albicans* was also thus efficiently achieved by this molecular modelling technique.

Amidst the *SAP* family, *SAP-1* was selected to interact with *C. cassia* biocompounds as it was freely retrieved from the PDB database as a desirable target based on the specific chemistry of the protein recorded in database. Accordingly, cinnamaldehyde, cinnamoyl acetate, eugenol and the control itraconazole docked against *Sap-1* of *C. albicans* resulted in a promising receptor – ligand complex. Docking analysis involved two major steps where the prediction of the exact orientation of the conformers in to the best active site pocket called pose and the strength of target-ligand binding interactions was achieved by scoring [20]. Analysis using Acceryls Discovery studio visualizer to predict hydrogen bond interactions between *SAP-1* and the *C.cassia* bio-compounds yielded promising results with hydrogen bonds and bonding energies. The number of hydrogen bonds together with the enthalpic gain due to the water molecules determines the best fit [21]. In this context, cinnamoyl (E) acetate scores to be the best inhibitory agent of *SAP-1* with a docking score of –5.33 Kcal/mol with 4 hydrogen bonds albeit, itraconazole with a score of –8.05 Kcal/mol but with one hydrogen bond. This is followed by eugenyl acetate with a score of –5.21 Kcal/mol again with 4 hydrogen bonds.

To assess the drug-ligand interactions between *C.cassia* bio-compounds and *Sap1*, we used auto dock tool 4.2 which is considered as a suite of automated docking tool with a software for modelling flexible small molecule such as drug molecule binding to receptor proteins of known three dimensional structure. Using genetic algorithms for the conformational search a rapid grid based method of energy evaluation was achieved in the study in analyzing the docking simulations. In this study, the Lamarckian Genetic Algorithm (LGA) was used to explore the binding conformational landscape of cinnamaldehyde, cinnamoyl acetate, eugenol and the control itraconazole docked against *Sap-1* of *C. albicans*. The docking scores of *C. cassia* bio-compounds on *Sap-1* indicated that there is a direct relationship between the energy of the binding affinity, referring to the lowest docking scores and the stability. In accordance with this, apart from the binding energy, the inter-molecular energy, vanderwaal's energy and torsional energy were also at a higher end for eugenyl acetate followed by cinnamoyl acetate, recording their anti-fungal medicinal value.

We performed molinspirational calculations in the present study to assess and evaluate the drug likeliness of the selected *C. cassia* bio-compounds. This is due to the fact that molecular properties such as membrane permeability, hydrophobicity and bioavailability are associated with some basic molecular descriptors such as log P [partition coefficient], log S [solubility], molecular weight, number of hydrogen bond acceptors and donors in a molecule are attributed to the concept of drug likeliness of the ligands and has wide acceptance in novel drug discovery and development [22]. In the present study, molinspirational results were very promising for cinnamaldehyde, cinnamoyl acetate and eugenol with the n-violations as zero satisfying the Lipinsky's rule of five with 3 violations for the control itraconazole suggesting the promising *SAP-1* inhibitory activity of the selected compounds from *C. cassia*, for further consideration towards drug designing and product development.

In molinspiration analysis, topological polar surface area (TPSA) of a molecule is considered as a useful descriptor to characterize the drug absorption and bio-availablity. The values of TPSA and OH-NH interactions in the present study, indicate that the selected ligands viz, cinnamaldehyde, cinnamoyl acetate and eugenol to possess a smooth and efficient binding to the target protein. It is predicted that the drug molecules with TPSA values of >140 Å or higher have low-absorption with the lipophilicity (miLogP) and have a vital role in the oral bioavailability for the drugs. In this context, *C.cassia* bio-compounds scored high absorption with high membrane penetration with a TPSA score of <140 Å, emphasizing their suitability for medicinal drugs to combat the menace of candidal infections.

Table 4e. Overall docking results of *C. cassia* biocompounds with SAP-1

SAP 1 docking with compounds	Number of hydrogen bonds	Binding energy	Ligand efficiency	Inhibition Constant (μM)	Inter-molecular energy	vdW + Hbond + desolv Energy	Electrostatic energy	Torsional energy	Total internal Unbound
(E)-Cinnamaldehyde	1	–4.91	–0.49	252.39	–5.51	–5.43	–0.07	0.6	–0.14
Cinnamoyl (E)-acetate	4	–5.33	–0.38	123.83	–6.52	–6.3	–0.22	1.19	–0.37
Eugenyl acetate	4	–5.21	–0.35	151.3	–6.7	–6.4	–0.31	1.49	–0.56
Itraconazole	1	–8.05	–0.16	1.25	–11.33	–11.32	–0.01	3.28	–2.78

4. CONCLUSION

Novel selection of inhibitors against specific target protein against *C. albicans* is considered for chemotherapy in recent years by computational assessment [23,24]. The docking calculations in this study suggest the promising medicinal value of *C. cassia* bio-compounds, cinnamaldehyde, cinnamoyl acetate and eugenol from *C. cassia* against the *SAP-1* of *C. albicans*. The preliminary clue obtained from the present investigation alarms for further target based experimental screening of the *C. cassia* bio-compounds for better selectivity and mechanism of action towards emphasizing its potent medicinal value in eradicating candidal infections.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sdiarticle4.com/review-history/59708